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ORIGINAL ARTICLE

Bio-based biodegradable hydrogels prepared by crosslinking of microbial poly(γ-glutamic acid) with L-lysine in aqueous solution

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Bio-based biodegradable hydrogels were prepared by crosslinking microbial homo poly(amino acid), poly(γ-glutamic acid) (PGA), with L-lysine in a one-pot synthesis in the presence of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) at 25 °C. The crosslinking was carried out in aqueous solution and the yields of the gels prepared with DMT-MM were higher than those prepared with water-soluble carbodiimide. The water absorption of the PGA gels crosslinked with L-lysine ranged from 300 to 2100 g/g. The water absorption of the PGA gel was affected by the amount of crosslinker and DMT-MM, which had an effect on the crosslinking density of the PGA gel. It was confirmed in a hydrolysis test in a buffer of pH 9 at 37 °C that the obtained gel that was crosslinked with L-lysine by amide bonds was more resistant to hydrolysis than the gel crosslinked by ester bonds. biochemical oxygen demand-biodegradability of the PGA gel crosslinked with L-lysine reached 60% in activated sludge at 25 °C.
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Keywords: biodegradable; crosslinking; hydrogels; poly(γ-glutamic acid); water absorption

INTRODUCTION

In recent years, it has been widely recognized that green chemistry is a very important concept for sustainable development. The use of renewable bio-based materials promises to be one of the solutions for global sustainability. For example, bio-based poly(L-lactide), chemically synthesized from L-lactic acid obtained by the fermentation of sugar, is used in various fields such as plastic food containers and automobile parts as an alternative to petroleum-based polymers for reduction of carbon dioxide emissions. There are also some biopolymers directly obtained by fermentation, for example, poly(hydroxyalkanoate)s,[1] poly(ε-lysine)[2] and poly(γ-glutamic acid) (PGA).[3] Modifications of poly(amino acid)s such as PGA have been studied in order to develop high-performance polymers because they are biodegradable and have functional groups. PGA is a polymer produced by some microorganisms such as *Bacillus subtilis* F-2-01[4] and *Bacillus subtilis* IFO3335.[5] Crosslinked PGA gels were reported by many researchers for medical or environmental applications, such as drug-release systems[6–10] and water treatment reagents.[11] Crosslinked PGA gels were prepared using γ-irradiation[6,7,11,12] or crosslinking reagents, such as dihalogenoalkanes,[8,9] alkyldiamines[10] and diisocyanates.[13] Crosslinking reagents are generally more useful than γ-irradiation because special facilities are not required. However, the currently available crosslinkers used in most of the studies are compounds of petroleum origin. With respect to green chemistry, it is desirable to have not only the main chains of the gel but also the crosslinkers to be made from bio-based materials.

We previously reported the preparation of bio-based hydrogels by crosslinking microbial PGA with various saccharides using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSC, water-soluble carbodiimide) in dimethyl sulfoxide (DMSO).[14] However, from the standpoint of green chemistry, it is considered that water is a better reaction solvent than other organic solvents because of low toxicity.[15] In our previous paper,[14] PGA was not crosslinked using WSC in water by ester bond with neutral sugars such as α-cyclodextrin (α-CD), but crosslinked by amide bond with amino sugars or basic amino acids such as chitosan or L-lysine. For example, crosslinking of PGA by water-soluble chitosan using WSC occurred in both DMSO and water, although the yield in DMSO was much higher than in water.[14] Therefore, we investigated the crosslinking method to obtain a PGA gel by amide bond in a high yield in water. Kunishima *et al.* have reported that the condensing agent, 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), could be used for formation of amide linkages in water in good recoveries.[16,17] We now report PGA gels crosslinked with L-lysine using DMT-MM in water. A PGA gel crosslinked by an amide bond with L-lysine is expected to be more resistant to hydrolysis than the gel by ester bond. Therefore, hydrolysis and biodegradation of the gels were also investigated.

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EXPERIMENTAL PROCEDURE

Materials and measurements

PGA was kindly gifted by Meiji Seika Kaisha, Ltd (Tokyo, Japan). The molecular weight of the PGA was $3.1\times10^5\,g\,mol^{-1}$ from the information provided by Meiji Seika Kaisha, Ltd WSC, L-lysine monohydrochloride (L-lysine·HCl) and L-ornithine monohydrochloride (L-ornithine·HCl) were purchased from Wako Pure Chemical Co., Ltd (Osaka, Japan). DMSO was purchased from Junsei Chemical Co., Ltd (Tokyo, Japan). DMT-MM was synthesized as reported by Kunishima *et al*.[16] All the reagents were reagent grade and used as received.

The ^1H nuclear magnetic resonance (NMR) spectra of the hydrolysates of the PGA gels were recorded on a JNM-GSE400 NMR spectrometer (JEOL, Ltd, Tokyo, Japan) operating at 400 MHz. The signal of H_2O at 4.80 p.p.m. (from 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt) was used as an internal reference. The PGA solutions were prepared as follows: the insoluble PGA gel was placed in a test tube and then D_2O was added. The pH of the solution was adjusted to 10 with NaOD/D_2O solution. The mixture was stored overnight at 60 °C. The PGA gel dissolved gradually as hydrolysis proceeded. The ^1H NMR spectra of the solution of the PGA gel were then measured.

The amount of L-lysine contained in the PGA gels was determined by high-performance liquid chromatography.[18] The PGA gel was weighed and transferred into test tubes to hydrolyze into amino acids by hydrochloric acid. They were dried after neutralization, and modified into phenylthiocarbamyl amino-acid derivatives. The amount of each phenylthiocarbamyl-amino-acid derivative was measured by a Waters Pico-Tag amino acid analyzing system equipped with a Waters Pico-Tag method column, two Waters 510 HPLC pumps and a Waters 486 tunable absorbance detector (Waters Corp., Milford, MA, USA).

The viscoelastic properties of the PGA gels were measured by an ARES viscoelastic measurement system (TA Instruments, Co., Ltd, New Castle, DE, USA). The dynamic frequency sweep tests were conducted in the range of 0.10–15 Hz using 25-mm diameter parallel plates at a constant value of shear strain (3%) at 24 °C.

The hydrolysis of the PGA gel in boric acid–borax buffer solution (0.1 M, pH 9) was monitored by size-exclusion chromatography (Waters 650 system) using size-exclusion chromatography columns (Waters Protein-Pak 300SW×2). The system was calibrated with protein standards. The dissolved PGA was detected at 220 nm with a Waters 486 tunable absorbance detector. Tris-HCl buffer solution (50 mM, pH 7.5) was used as an eluent at $0.50\,ml\,min^{-1}$.

Crosslinking of PGA by WSC in water and DMSO

The methods to prepare PGA gels with L-lysine are shown in Scheme 1. In the method of Scheme 1a, DMSO was used as a reaction solvent. A 100-mg (0.77 mmol of glutamic acid residue) sample of PGA was transferred into a glass test tube, and 0.70 ml of DMSO was added. The contents of the tube were dissolved in DMSO by ultrasonication. An 80 mg (0.42 mmol) sample of WSC dissolved in 0.30 ml of DMSO and 35.3 mg (0.19 mmol) of L-lysine·HCl dissolved in 0.16 ml of water and 1.0-M NaOH to adjust the pH (pH 5.6–9) were added to the PGA solution in that order. The molar ratio of L-lysine to glutamic acid residue in PGA was 0.25 in this case. The mixture was stirred at 25 °C. After 24 h, an excess amount of acetone was added to the test tube and the reaction product was isolated as a precipitate by decantation. The product was then kept in phosphate buffer of pH 7 overnight, and then 1.0-M NaOH was added to adjust the pH of the solution to 8. The swelled PGA gel was transferred into a nylon mesh bag (mesh 255, 57 μm open pitch) and filtered. The PGA gel in the bag was washed by dipping the bag in distilled water and changing the water once a day for a week. After removal of excess water, the swelled gel was lyophilized to obtain the dry PGA gel. Yield of the dry PGA gel was calculated using the following equation:

$$\text{Yield}\,(\%) = W_{gel}/W_{PGA}\times100 \quad (1)$$

where W_{gel} is the weight of the dry PGA gel and W_{PGA} is the weight of the dry PGA before crosslinking.

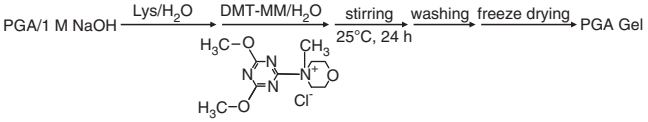
The dry gel contains PGA sodium salt and crosslinker moiety of L-lysine; therefore, the yield may exceed 100% as the crosslinking reaction proceeds.

In the method described in Scheme 1b, water was used instead of DMSO as a reaction medium. A 50 mg (0.39 mmol) sample of PGA was placed in a test tube and dissolved in 0.19 ml of 1.0-M NaOH. A measure of 40 mg (0.21 mmol) of WSC powder and 17.7 mg (0.097 mmol) of L-lysine·HCl dissolved in 0.08 ml of water and 1.0-M NaOH to adjust the pH (pH 5.6–9.7) were added to the reaction mixture in that order. The molar ratio of L-lysine to glutamic acid residue in PGA was 0.25, similar to the crosslinking in DMSO. The mixture was stirred at 25 °C for 24 h. The product was washed and dried by the method described above.

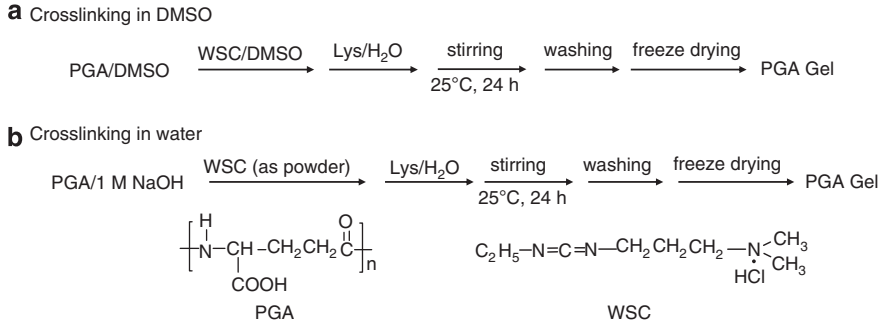
A PGA gel crosslinked by α-CD was also prepared using WSC in DMSO in accordance with the procedure in the reference.[14] The molar ratio of α-CD added to glutamic acid residue in PGA was 0.25 and the obtained PGA gel was used for a hydrolysis test described later.

Crosslinking of PGA with L-lysine by DMT-MM in water

The preparation method of the PGA gel using DMT-MM is shown in Scheme 2. The typical procedure is as follows: a 50 mg (0.39 mmol) sample of PGA was placed in a test tube and dissolved in 0.35 ml of 1.0-M NaOH. A measure of 17.7 mg (0.097 mmol) of L-lysine·HCl dissolved in 0.08 ml of water and 1.0-M NaOH was added to adjust the pH to 8.9 and the mixture was stirred for 0.5 h. The molar ratio of L-lysine to glutamic acid residue in PGA was 0.25. Thereafter, a 54 mg (0.20 mmol) sample of DMT-MM dissolved in 0.20 ml of water was transferred into the test tube and the mixture was stirred at 25 °C for 24 h. The product was washed and dried by the method described above. The obtained gel was used for a hydrolysis



Scheme 2 A method for crosslinking of poly(γ-glutamic acid) (PGA) with L-lysine by 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) in water.



Scheme 1 Methods for crosslinking of poly(γ-glutamic acid) (PGA) with L-lysine by water-soluble carbodiimide (WSC) in dimethyl sulfoxide (DMSO) and water.

test and biochemical oxygen demand (BOD)-biodegradation described later. Yield of the dry PGA gel was calculated using Equation (1). The ^{1}H NMR spectrum of the solution of the hydrolysate of the PGA gel crosslinked with L-lysine by DMT-MM was as follows. The underlined characters represent the assigned protons: δ=1.31 (m, 2H, NHCH$_2$CH$_2$C<u>H$_2$</u>CH$_2$CH), 1.41 (m, 2H, NHCH$_2$CH$_2$CH$_2$C<u>H$_2$</u>CHCOO), 1.51 (m, 2H, NHC<u>H$_2$</u>C<u>H$_2$</u>CH$_2$CH$_2$CHCOO), 1.92, 2.04 (m, 2H, O(O=)CCH$_2$C<u>H$_2$</u>CHCOO), 2.35 (m, 2H, O(O=)CC<u>H$_2$</u>CH$_2$CHCOO), 2.58 (m, 2H, NHC<u>H$_2$</u>CH$_2$CH$_2$CH$_2$CHCOO), 3.20 (m, 1H, NHCH$_2$CH$_2$CH$_2$CH$_2$C<u>H</u>COO), 4.12 (m, 1H, O(O=)CCH$_2$CH$_2$C<u>H</u>COO).

The values of the storage modulus (G') of the PGA gels at 1.0 Hz were measured for the evaluation of the crosslinking densities of the gels. The gel samples were prepared in the parallel plates by crosslinking of PGA with different amounts of L-lysine by DMT-MM for 24 h and used for the measurements without washing. The PGA gels in the parallel plates were about 0.7 mm thick and contained ~9 g of water in 1 g of the PGA gel.

Water absorption of PGA gels

The dried PGA gels were weighed and transferred into the same type of nylon mesh bag as used for washing the gel. The bag was then dipped in distilled water at 25 °C. After 24 h, it was hung for 10 min to remove excess water and weighed. To eliminate water absorption of a nylon mesh bag, the same operation for the empty nylon bag was carried out as the reference. Water absorption (gram per gram) of the PGA gel was calculated from the following equation:

$$\text{Water absorption} = (Q_1 - Q_0)/Q_0 \quad (2)$$

where Q_0 is the weight of the dry PGA gel and Q_1 is the weight of the swelled PGA gel.

Hydrolysis of PGA gels

Hydrolysis of the PGA gel crosslinked with L-lysine and the gel crosslinked with α-CD was examined to compare hydrolysis of PGA gels among different types of crosslinkers. L-Lysine was attached to PGA in amide bond and in contrast α-CD was attached to PGA in ester bond. The molar ratio of the crosslinker, L-lysine or α-CD, to glutamic acid residue in each PGA gel was 0.25, and the preparations of each PGA gel have been described in the previous sections. The gels were added at a concentration of 2.0 mg ml^{-1} to a buffer solution of pH 9 (0.1 M boric acid–borax) at 37 °C. The concentration of PGA in the supernatant was periodically monitored by size-exclusion chromatography analysis using the 2.0 mg ml^{-1} PGA solution as a standard. The maximum concentrations of PGA were 1.5 mg ml^{-1} for the PGA gel crosslinked by amide bond with L-lysine and 1.4 mg ml^{-1} for the gel crosslinked by ester bond with α-CD. Hence, the degree of hydrolysis of PGA was calculated from the following equation:

$$\text{Degree of hydrolysis} = C_1/C_0 \quad (3)$$

where C_0 is the maximum concentration of PGA (1.5 mg ml^{-1} for the PGA gel crosslinked with L-lysine and 1.4 mg·ml^{-1} for the gel crosslinked with α-CD) and C_1 is the concentration of PGA in the supernatant.

Measurement of biodegradability of PGA gels

Biodegradability of the PGA gel crosslinked with L-lysine was evaluated by a BOD test, according to the OECD Guidelines for Testing Chemicals (301C, modified MITI test). BOD was determined with a BOD tester (Denkikagaku-keisoku Corp., Tokyo, Japan). A culture medium for the test was prepared by dissolving 85 mg of KH_2PO_4, 217.5 mg of K_2HPO_4, 334 mg of $Na_2HPO_4{\cdot}2H_2O$, 5 mg of NH_4Cl, 22.5 mg of $MgSO_4{\cdot}7H_2O$, 36.4 mg of $CaCl_2{\cdot}2H_2O$ and 0.25 mg of $FeCl_3{\cdot}6H_2O$ in 1 l of distilled water and the pH of the culture medium was 7.4. The molar ratio of L-lysine to glutamic acid residue in the PGA gel used for the test was 0.25, and the preparation of the gel was described in the previous section. A 30 mg sample of PGA gel and 300 ml of the culture medium was transferred into a bottle of the BOD tester. Activated sludge from a sewage plant was used as a microorganism source and 9 mg of suspended solid in the activated sludge was added into the bottle. A bottle with 30 mg of aniline instead of a PGA gel as a control and one without organic matter as a blank were also prepared. The bottles were set in the BOD tester and stirred at 25 °C. BOD-biodegradability (BOD/ThOD) was calculated from the BOD values and the theoretical oxygen demand (ThOD).

RESULTS AND DISCUSSION

Crosslinking of PGA with L-lysine by WSC in DMSO and water

PGA was crosslinked by condensation between the carboxyl groups of PGA and the amino groups of L-lysine. This reaction occurred in the presence of WSC as a condensing agent in DMSO (a in Scheme 1) and water (b in Scheme 1). The results of crosslinking of PGA with L-lysine by WSC in DMSO (Figure 1) and in water (Figure 2) by using aqueous L-lysine solutions at various pH values were compared. As shown in Figure 2, the maximum yield in water was 38%. This value is almost the same as the maximum yield of crosslinked PGA with 1,3-diaminopropane in water (39.9 mg of PGA gel from 100 mg of PGA and 25 µl of 1,3-diaminopropane), as reported by Kunioka and Furusawa.[10] They indicated that the low yield might be because of the low reactivity of PGA and WSC into PGA–WSC adducts, and also because of the low reactivity of PGA–WSC adducts and 1,3-diaminopropane into a PGA gel.[10]

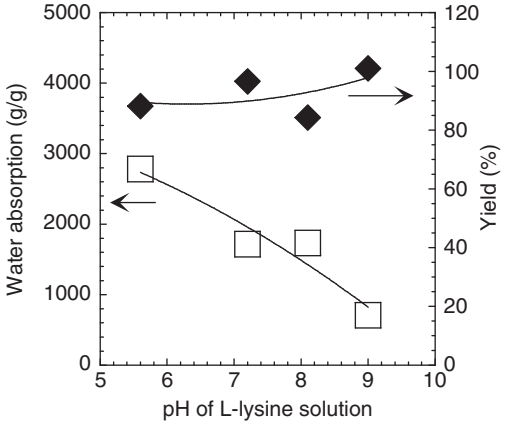


Figure 1 Crosslinking of poly(γ-glutamic acid) (PGA) in dimethyl sulfoxide (DMSO) by water-soluble carbodiimide (WSC). Relationship between pH of L-lysine solution and water absorption (□) or yield (◆) of the obtained PGA gel. PGA gels were prepared with 100 mg of PGA dissolved in 0.70 ml of DMSO, 35.3 mg of L-lysine·HCl in 0.16 ml of water and 1.0 M NaOH to adjust the pH, and with 80 mg of WSC dissolved in 0.30 ml of DMSO at 25 °C for 24 h.

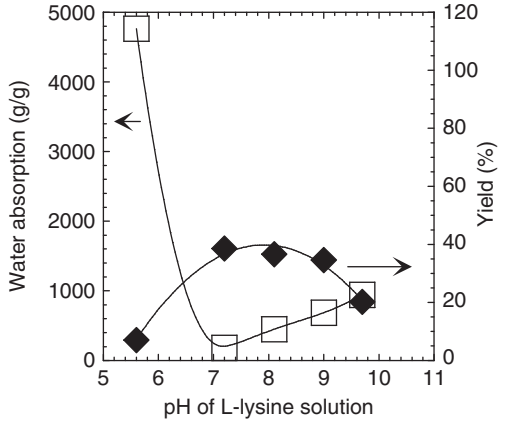
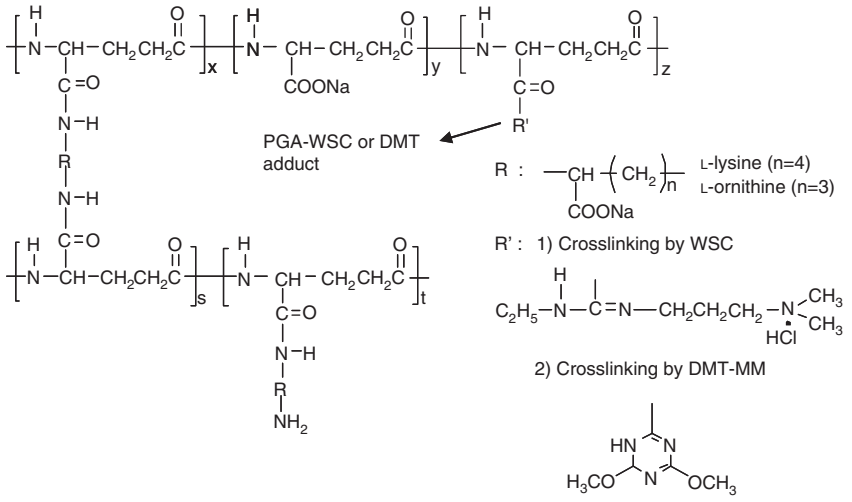


Figure 2 Crosslinking of poly(γ-glutamic acid) (PGA) in water by water-soluble carbodiimide (WSC). Relationship between pH of L-lysine solution and water absorption (□) or yield (◆) of the obtained PGA gel. The PGA gels were prepared with 50 mg of PGA dissolved in 0.19 ml of 1.0-M NaOH, 17.7 mg of L-lysine·HCl in 0.08 ml of water and 1.0-M NaOH to adjust the pH, and with 40 mg of WSC powder at 25 °C for 24 h.



Scheme 3 Possible structure of poly(γ-glutamic acid) (PGA) gels crosslinked by L-lysine or L-ornithine in the presence of water-soluble carbodiimide (WSC) or 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM).

On the other hand, yields of the reaction in DMSO were more than 80% and remained almost constant when the pH of L-lysine solution varied from 5.6 to 9.0 as shown in Figure 1. However, water absorption of the PGA gel was higher when the PGA gel was prepared using L-lysine solution at a lower pH. The high water absorption was attributed to the low crosslinking density. It is suggested that the PGA gel, prepared by using L-lysine solution of a low pH, had a less crosslinked structure. The possible structure of PGA gel is shown in Scheme 3. Comparing Figures 1 and 2, yield of the reaction in water was lower than that in DMSO. When WSC was used for crosslinking of PGA by L-lysine, the best result was obtained by the reaction in DMSO using pH 5.6 L-lysine solution, with respect to the yield and water absorption (88% and 2900 g/g, respectively). To investigate the effect of the alkyl chain length between two amino groups, L-ornithine also reacted with PGA to form gels in DMSO by WSC. When PGA was crosslinked under the condition that the molar ratio of L-ornithine to glutamic acid residue of PGA was 0.25 at pH 9, the yield and water absorption of the PGA gel were 89% and 1400 g/g, respectively. The yield and water absorption of the gel crosslinked with L-lysine under the same condition was 101% and 710 g/g, respectively. Comparing these results, L-lysine was more reactive for crosslinking with PGA by using WSC than was L-ornithine. Because L-lysine has one methylene longer chain between two amino groups than does L-ornithine, it is thought that amino groups in L-lysine can more readily access the carboxyl groups of the PGA to form intermolecular crosslinking compared with those of L-ornithine.

Crosslinking of PGA with L-lysine by DMT-MM in water

The crosslinked PGA by L-lysine was obtained in water by using the conventional condensing agent, WSC, as described above. However, WSC is less reactive in water, which is a desirable solvent from the standpoints of the environment and safety.[15] To achieve crosslinking of PGA by L-lysine in water with a higher yield, we used another condensing agent, DMT-MM, reported by Kunishima *et al.*[16,17] As DMT-MM could be efficiently used for formation of amide bonds in water,[16] we used it for crosslinking in this study.

Figure 3 shows the results of crosslinking of PGA with L-lysine by DMT-MM in water. PGA gels were prepared with 50 mg (0.39 mmol of glutamic acid residue) of PGA and 17.7 mg (0.097 mmol) of L-lysine·HCl. The reaction was carried out at various pH values of the

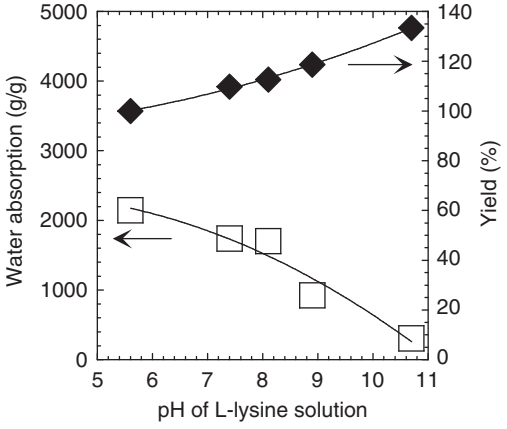


Figure 3 Crosslinking of poly(γ-glutamic acid) (PGA) by 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM). Relationship between pH of L-lysine solution and water absorption (□) or yield (◆) of the obtained PGA gel. The PGA gels were prepared with 50 mg of PGA dissolved in 0.35 ml of 1.0-M NaOH, 17.7 mg of L-lysine·HCl dissolved in 0.08 ml of water and 1.0-M NaOH to adjust the pH, and with 54 mg of DMT-MM dissolved in 0.20 ml of water at 25 °C for 24 h.

L-lysine solution. The yield exceeded 100% because the recovered weight of the sample was increased by L-lysine binding to PGA and by addition of sodium ion to the carboxyl groups of PGA for neutralization. For example, when the yield was 133% (66.7 mg of the PGA gel) at pH 10.7 of L-lysine solution in Figure 3, the amount of L-lysine that bound to 50 mg of PGA was calculated to be 12.0 mg (0.082 mmol) from the amino-acid composition determined by high-performance liquid chromatography, and the rest of the PGA gel weight, 4.7 mg, was attributed to the sodium ion added to PGA for dissolution and neutralization. Water absorption of the PGA gel was higher when lower pH values of L-lysine solution were used for the reaction, and this result was similar to that of the reaction by WSC in DMSO as shown in Figure 2. High water absorption may be caused by the low crosslinking density. Kunishima *et al.* reported that the reactions by DMT-MM generally proceeded under almost neutral or weak alkaline conditions. They concluded that pretreatment of the carboxylic acid and the amine to be condensed, to form the ammonium carboxylate

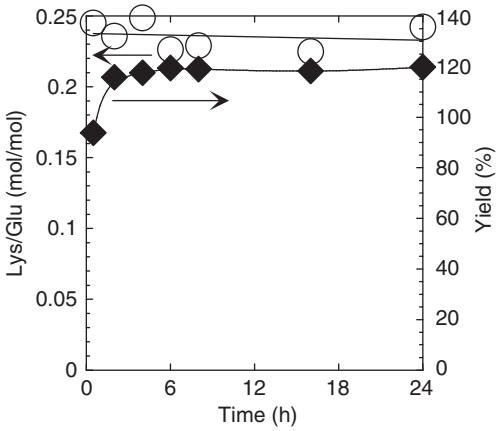


Figure 4 Time course of crosslinking by 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM). Molar ratio of L-lysine to glutamic acid residue (Lys/Glu) (○) or yield (◆) of the obtained poly(γ-glutamic acid) (PGA gel). The PGA gels were prepared with 50 mg of PGA dissolved in 0.35 ml of 1.0-M NaOH, 17.7 mg of L-lysine·HCl dissolved in 0.08 ml of water and 1.0-M NaOH to adjust the pH to 8.9, and 54 mg of DMT-MM dissolved in 0.20 ml of water at 25 °C. Values of Lys/Glu in the obtained PGA gels were determined by high-performance liquid chromatography.

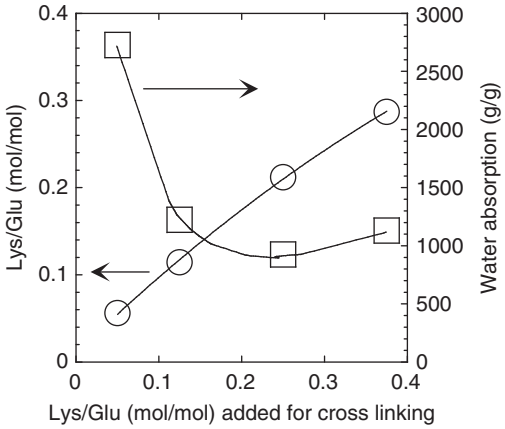


Figure 5 Relationship between the molar ratio of L-lysine to glutamic acid residue (Lys/Glu) added for crosslinking and Lys/Glu in the obtained poly(γ-glutamic acid) (PGA) gel (○) or water absorption (□). The PGA gels were prepared with 50 mg of PGA dissolved in 0.35 ml of 1.0-M NaOH, L-lysine·HCl dissolved in 0.08 ml of water and 1.0-M NaOH to adjust the pH to 8.9, and 54 mg of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride dissolved in 0.20 ml of water at 25 °C for 24 h. Values of Lys/Glu in the obtained PGA gels were determined by high-performance liquid chromatography.

salt, would have promoted the activation of the carboxylic acid by DMT-MM, because the reaction rates of carboxylate anions were likely to be much faster than those of free carboxylic acids.[16] Because the carboxylate anions on PGA in the reaction mixture decreased with decreasing the pH of L-lysine solution, the reaction rate decreased to make the crosslinking density lower.

Compared with Figure 2, the yields of gels by DMT-MM in Figure 3 were much higher than those by WSC in the pH range of L-lysine solution used for crosslinking. This means that DMT-MM is more reactive than WSC in water and can be used for crosslinking in water more efficiently.

Figure 4 shows the time course of crosslinking by DMT-MM. The L-Lysine content of the gel was constant, but yield was relatively low at the beginning of the reaction. This is because some PGA chains were not crosslinked enough to become insoluble in water and some parts of the gel were washed off from the insoluble gel at the beginning of the reaction.

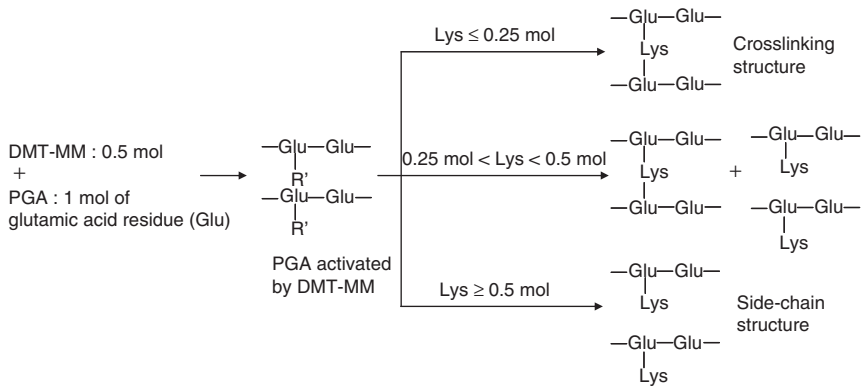
Effect of the amount of L-lysine and DMT-MM on L-lysine content in PGA gels and water absorption

Figure 5 shows the effect of the amount of L-lysine added for crosslinking of PGA on L-lysine content in the obtained PGA gels and on water absorption. It was revealed that the molar ratio of L-lysine to glutamic acid residue in the obtained PGA gels (Lys/Glu on the left *y* axis) increased in proportion to the molar ratio of L-lysine to glutamic acid residue added for crosslinking (Lys/Glu used for crosslinking on the *x* axis). Water absorption reached a minimum when Lys/Glu on the left *y* axis was 0.21 and slightly increased when it was 0.29.

Scheme 4 shows structures formed by the reaction using 1 mol of glutamic acid residue in PGA, 0.5 mol of DMT-MM and a different molar ratio of L-lysine. Considering the reaction mechanism of DMT-MM,[16] 0.5 mol of the carboxyl group in the glutamic acid residue of PGA is activated by DMT-MM. As an activated carboxyl group reacts with one of two amino groups in L-lysine, L-lysine less than half the amount of activated carboxyl groups (Lys $\leqslant$ 0.25 mol) reacts with PGA to form a crosslinking structure. On the other hand, when the amount of L-lysine is more than half the amount of activated carboxyl group (Lys $>$ 0.25 mol), L-lysine partly reacts with PGA only by one amino group and forms a side-chain structure. In the series of experiments, the results of which shown in Figure 5, a 0.20 mmol of DMT-MM and 0.39 mmol of glutamic acid residue in PGA were used, and 0.20 mmol (0.51 M ratio) of the carboxyl group in the glutamic acid residue of PGA was activated by DMT-MM at a maximum. When the molar ratio of L-lysine to glutamic acid residue of the obtained PGA gel (Lys/Glu on the left y-axis) was 0.29, this value exceeded 0.25, which was half the molar ratio of the activated carboxyl group of PGA. Hence, some of L-lysine was thought to bind to PGA by only one amino group as a side chain and does not function as a crosslinker. As a result, the crosslinking density became low and water absorption of the PGA gel increased when Lys/Glu on the left *y* axis was 0.29 compared with 0.21.

The values of G' of the PGA gels prepared under the corresponding conditions of the gels with 0.11, 0.21 and 0.29 of Lys/Glu on the left *y* axis of Figure 5 were 250, 630 and 530 Pa, respectively. The PGA gel with 0.21 of Lys/Glu showed a maximum value of G' and a minimum value of water absorption. Both results indicated that the PGA gel with 0.21 of Lys/Glu had the highest crosslinking density among the obtained gels.

Figure 6 shows the effect of the amount of DMT-MM added for crosslinking on L-lysine content in the obtained PGA gels and on water absorption. Lys/Glu on the left y axis is the molar ratio of L-lysine to glutamic acid residue in the obtained PGA gels. The L-Lysine content in PGA gels was not affected significantly by changing the amount of DMT-MM. However, when the amount of DMT-MM was low (DMT-MM/Glu on *x* axis=0.25), the yield was very low and water absorption was very high (7000 g/g). In Figure 6, the molar ratio of the amino group in L-lysine added to the carboxyl group in PGA was 0.5; hence, only half the amount of the amino group in L-lysine could react with PGA when DMT-MM/Glu was 0.25. Under this reaction condition, PGA and L-lysine mainly formed a side chain similar to the structure shown in Scheme 4. When DMT-MM/Glu was 0.25, the gel was obtained in low yield and was very fragile, probably because of the very low crosslinking density, which was not enough to make PGA insoluble.



Scheme 4 Structures formed by the reaction using 1 mol of glutamic acid residue in poly(γ-glutamic acid) (PGA), 0.5 mol of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) and different molar ratio of L-lysine. R′: carboxyl group activated by DMT-MM.

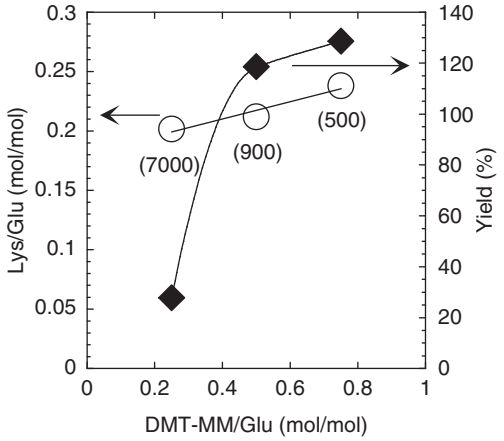


Figure 6 Relationship between the molar ratio of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) to glutamic acid residue added for crosslinking and molar ratio of L-lysine to glutamic acid residue (Lys/Glu) (○) or yield (◆) of the obtained poly(γ-glutamic acid) (PGA) gel. The PGA gels were prepared with 50 mg of PGA dissolved in 0.35 ml of 1.0-M NaOH, 17.7 mg of L-lysine·HCl dissolved in 0.08 ml of water and 1.0-M NaOH to adjust the pH to 8.9, and DMT-MM dissolved in 0.20 ml of water at 25 °C for 24 h. Values of Lys/Glu in the obtained PGA gels were determined by high-performance liquid chromatography. Numbers in parentheses are water absorptions (g/g) of the obtained PGA gels.

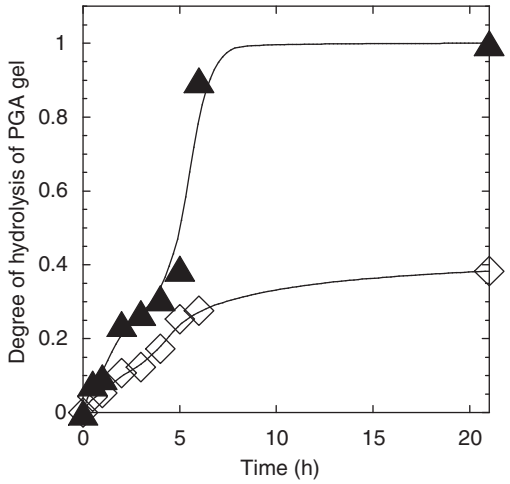


Figure 7 Hydrolysis of the poly(γ-glutamic acid) (PGA) gels in 0.1-M boric acid–borax buffered solution (pH 9) at 37 °C. The PGA gel crosslinked by amide bond with L-lysine (◇) or crosslinked by ester bond with α-cyclodextrin (▲).

Hydrolysis and BOD-biodegradability of PGA gels

To compare the different types of crosslinkages, hydrolyzability of PGA gels crosslinked with L-lysine or α-CD in 0.1-M boric acid–borax buffer (pH 9) at 37 °C was evaluated. Water absorptions of the PGA gel that was crosslinked with L-lysine and the gel crosslinked with α-CD after 1 h in the buffered solution were 73 and 50 g/g, respectively. PGA was gradually dissolved in the buffer solution as hydrolysis of crosslinking proceeded. The size-exclusion chromatography analysis revealed that the dissolved PGA had almost the same molecular weight as the PGA before crosslinking. This means that dissolution was due to cleavage of the crosslinking moiety and not of the PGA main chain. The results are shown in Figure 7. The crosslinking structure of the PGA gels was gradually hydrolyzed and the concentrations of the dissolved PGA were increased. The PGA gel with α-CD was fully hydrolyzed after 21 h, whereas the PGA gel with L-lysine did not dissolve in the solution up to the maximum concentration. The PGA gel crosslinked with L-lysine was more stable than the one with α-CD. This is because amide bonds of the PGA gel with L-lysine are more resistant to hydrolysis than are ester bonds of the PGA gel with α-CD. The results indicate that the hydrolysis can be controlled by changing the type of crosslinkage.

Biodegradability of the PGA gel that was crosslinked with L-lysine was evaluated by the BOD test. Water absorption of the PGA gel after 1 h in the culture medium was 280 g/g. As shown in Figure 8, it was confirmed that BOD-biodegradability of the gel reached 60%, which is the criterion of ready biodegradability according to the OECD 301C. The bio-based PGA gel that was crosslinked with L-lysine is considered to be biodegradable in nature and can be applicable to soil improvement for instance.

Conclusion

Bio-based biodegradable hydrogels were prepared by crosslinking microbial PGA with L-lysine in aqueous solution. The crosslinking reactions were carried out by using WSC or DMT-MM as condensing agents. In the presence of WSC, PGA gels were obtained in higher yield in DMSO compared with those in water. For crosslinking of PGA in water, DMT-MM was a more useful condensing agent than WSC because of the higher yield. The yield and water absorption of the PGA gel were affected by the pH of the L-lysine solution, the molar ratio of the crosslinker and by DMT-MM. The gel crosslinked by amide bonds

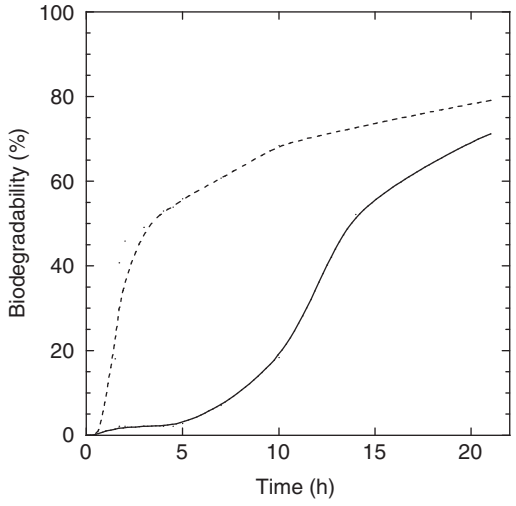


Figure 8 Biochemical oxygen demand-biodegradation test of the poly(γ-glutamic acid) (PGA) gel (solid line) and aniline (dashed line) using activated sludge at 25 °C. The PGA gel was prepared with 50 mg of PGA dissolved in 0.35 ml of 1.0-M NaOH, 17.7 mg of L-lysine·HCl dissolved in 0.08 ml of water and 1.0-M NaOH to adjust the pH to 8.9, and 54 mg of 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride dissolved in 0.20 ml of water at 25 °C for 24 h.

with L-lysine was more stable in a buffer of pH 9 at 37 °C than the gel crosslinked by ester bonds with α-CD. The PGA gel crosslinked with L-lysine was readily biodegraded in the BOD test. The PGA gel and the crosslinking method by DMT-MM will be applicable to the development of functional bio-based materials.

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